Immunocytochemical Localization of the Vitamin D-dependent Calcium Binding Protein in Chick Duodenum

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ABSTRACT The vitamin D-dependent calcium binding protein (CaBP) of chick duodenum has been localized by immunocytochemistry and by radioimmunoassay. Light microscopically, CaBP was seen to be present in the absorptive cells of the villi while in other cell types of the villi and the crypts, including goblet cells and endocrine cells, no CaBP was seen. At the electron microscopic level, CaBP was shown to be localized in the cytosol and the euchromatin of the nucleus but not in membrane-bounded cytoplasmic compartments. Quantitative evaluation of the immunocytochemical protein A-gold label showed that the terminal web and the cytosol of basal cellular regions were most highly labeled while the brush border was weakly labeled. The radioimmunoassay evaluation of intestinal subcellular fractions indicated that 96% of the homogenate CaBP is in the cytosol high-speed supernatant fraction. Collectively, these results support the hypothesis that the vitamin D-dependent intestinal CaBP may play a role in either regulation of intracellular calcium concentration or movement of calcium across the epithelial cell, rather than directly in the initial step(s) of calcium absorption across the brush border membrane from the gut lumen.

The intestinal absorption of calcium is dependent upon continuous access to vitamin D (calciferol) and its daughter metabolite 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] (29). 1,25-$(OH)_2D_3$ functions in a fashion analogous to that of classic steroid hormones to induce the biosynthesis of cellular components essential for an efficient calcium translocation process, including a calcium binding protein (CaBP, references 40, 43, 44). The steady-state level of intestinal CaBP is known to be affected by the dietary level of calcium and phosphorus (17). There is a good correlation between the absolute level of CaBP (1–3% of the soluble cellular protein) and both the occupancy of the intestinal 1,25$(OH)_2D_3$ receptors (2, 20) and the stimulation of intestinal calcium transport (24, 37).

Thus, there is little doubt that the vitamin D-dependent intestinal CaBP is associated with intestinal calcium uptake (28, 29) but the mechanism of its action at the cellular and molecular level and its localization within the intestinal mucosa are controversial. It has been suggested by Taylor and Wasserman (42) that CaBP is produced in goblet cells and located in the brush border of the absorptive columnar cells while others (1, 21, 25, 26) have found it to be present inside the absorptive columnar cells.

This controversy surrounding the localization and role of the vitamin D-dependent CaBP in the chick intestine and the lack of morphological data at the electron microscopic level prompted the present study. We found, by using light and electron microscopic immunolabeling techniques, that the vitamin D-dependent intestinal CaBP is present in the cytosol and euchromatin of the absorptive columnar epithelial cells. In addition, quantitative immunoelectron microscopy revealed a regional difference in the distribution of immunoreactive CaBP in the cytosol of the absorptive cell. This localization of vitamin D-dependent intestinal CaBP is compatible with a role for the protein in regulation of intracellular calcium concentration.

MATERIALS AND METHODS

Preparation of Tissue

Tissue was taken from 1-wk-old White Leghorn chicks. Pieces from the upper part of the duodenum were fixed in Bouin's fluid for 18 h at room temperature and embedded in paraffin by routine methods (light microscopy). Other pieces from the same region of the duodenum were fixed at room temperature for 2 h in 0.5% glutaraldehyde diluted in phosphate-buffered saline (PBS) for electron microscopy. After fixation, the tissue fragments were rinsed in PBS, and free aldehyde groups were blocked by incubation in 0.5 M NH_4Cl in PBS for 2 h at room temperature. After further rinses in PBS, the tissue pieces were dehydrated in a graded series of ethanol at room temperature and embedded in Epon 812. In

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addition to embedding in Epon, pieces of tissue fixed as described above were embedded at low temperature in Lowicryl K4M resin (8).

Thin sections of Epon- or Lowicryl K4M-embedded duodenum were cut with a diamond knife and picked up on 200-mesh, Parlodion-carbon-coated nickel grids.

Preparation of Antisera Against Chick Duodenal Calcium Binding Protein

Chick CaBP was prepared from the duodenum of 3-wk-old rachitic chicks given 32.5 nmol of vitamin D_3 72 h before sacrifice. It was purified to electrophoretic homogeneity as previously described (9). Purified chick CaBP was emulsified with Freund's complete adjuvant and used for immunization of New Zealand White rabbits (9). All rabbits produced antisera which gave a single precipitation line with intestinal mucosal homogenates from vitamin D_3-replete chickens in the Ouchterlony double diffusion test.

Immunocytochemical Procedures

For light microscopy, paraffin sections (5-μm thick) were stained by an indirect immunofluorescence method (4). Incubation was carried out with anti-CaBP serum at dilutions of 1:50–1:300 for 2 h at room temperature; the sections were then rinsed in PBS and further incubated with fluorescein-labeled protein A (0.1 mg/ml) for 1 h at room temperature. After several rinses in PBS, immunostained sections were counterstained with 0.01% Evan's blue in PBS for 2 min and examined with a Leitz Orthoplan microscope equipped with an HBO 200-high pressure mercury vapor lamp and a Ploemopak reflected light illuminator.

For electron microscopy, thin sections of Epon- or Lowicryl K4M-embedded duodenum were stained for CaBP with the protein A-gold (pAg) technique (32, 33). The pAg complex was prepared as previously described (32) using the method of Frens (16) for preparation of colloidal gold (particle diameter ~15 nm).

The thin sections were placed first for 5 min on a droplet of 0.5% ovalbumin in PBS and were then transferred to the anti-CaBP serum for 2 h at room temperature or for 16 h at 4°C. The antiserum was used at dilution of 1:100 in ovalbumin-PBS. The sections were washed with PBS (three changes) for 10 min and incubated for 1 h at room temperature with 1:20 dilution of the pAg solution. After rinses with PBS and distilled water, the thin sections were counterstained with uranyl acetate (7 min) and lead citrate (30 s) and examined with a Philips EM 300 microscope.

Specificity of the immunostaining in light and electron microscopy was tested by incubating sections in antigen-absorbed anti-CaBP (400 μg purified duodenal CaBP/1 ml of nondiluted antiserum), homologous nonimmune rabbit serum, or ovalbumin-PBS in replacement of the anti-CaBP serum.

Quantitation of the Immunoelectron Microscopic Label

The intensity of immunostaining was expressed as the number of gold particles per square micrometer in the brush border, terminal web, supranuclear and basal cytoplasm, and in the euchromatin of the absorptive cells and in the supranuclear cytoplasm of the goblet cells. For each cell compartment studied, 20 micrographs were taken at 11,000 times magnification, calibrated with a carbon grating replica (2,160 lines/mm). The surface of the compartments and the number of gold particles present over them were recorded on a graphic tablet (Tektronix, Inc., Beaverton, OR; type 4973) connected to a microprocessor system (IMSAI, type 8080) programmed to calculate the number of particles per unit area (μm^2) of the compartment (D. Bertrand and M. Amherdt, manuscript in preparation). Statistical comparison of the values was performed with the Student's t test. The remaining cell compartments (Golgi apparatus, mitochondria, lysosomes) were not evaluated quantitatively because they did not show labeling above nonspecific background observed in control sections.

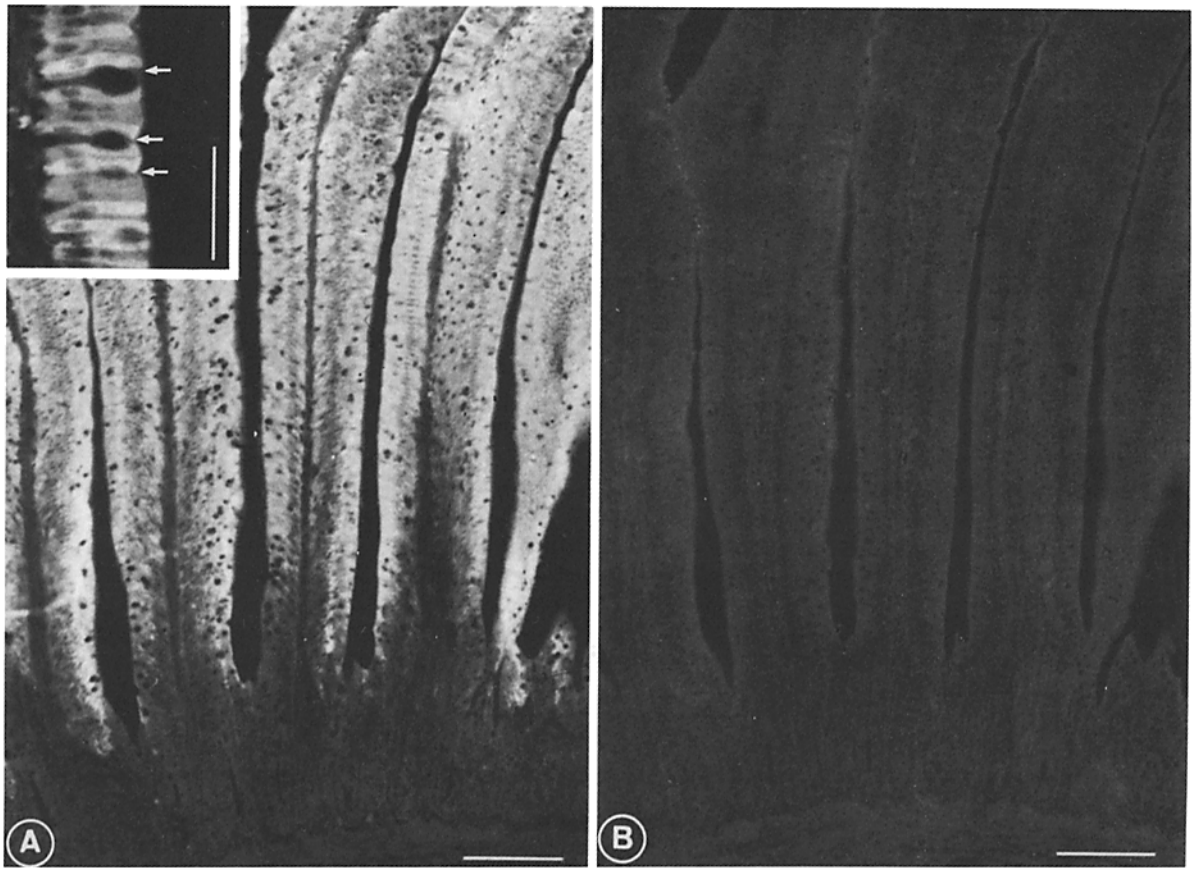


FIGURE 1 Immunofluorescence staining for CaBP in a paraffin section from chick duodenum (*A*) showing a positive reaction (*white*) in the absorptive cells on the villi. The goblet cells and the cells on the crypts as well as the stroma of the villi and the lamina propria are negative. The inset shows the varying staining intensity for CaBP in the absorptive cells. Note the lack of immunostaining in goblet cells (arrows). A paraffin section consecutive to *A* which was incubated with CaBP-antiserum absorbed with purified intestinal CaBP shows no immunostaining (*B*). *A* and *B*, × 130. Bars, 100 μm. Inset, × 300. Bar, 50 μm.

Radioimmunoassay for Vitamin D-dependent CaBP

The radioimmunoassay (RIA) procedure for the determination of the CaBP content of intestinal subcellular fractions was carried out exactly as described by Christakos et al. (9). All RIA's were carried out using a double antibody procedure which reduced background to a minimum and maximized sensitivity. This assay has a sensitivity of 1 ng, and interassay variability of 16% and intraassay variability of 4.0%. The protein concentration of all subcellular fractions was determined by the standard Lowry procedure (22).

Preparation of Intestinal Subcellular Fractions

Male White Leghorn cockerels (Pace Setter Hatchery, Alta Loma, CA) were fed our standard rachitogenic diet (29). After 3 wk each animal received 6.25

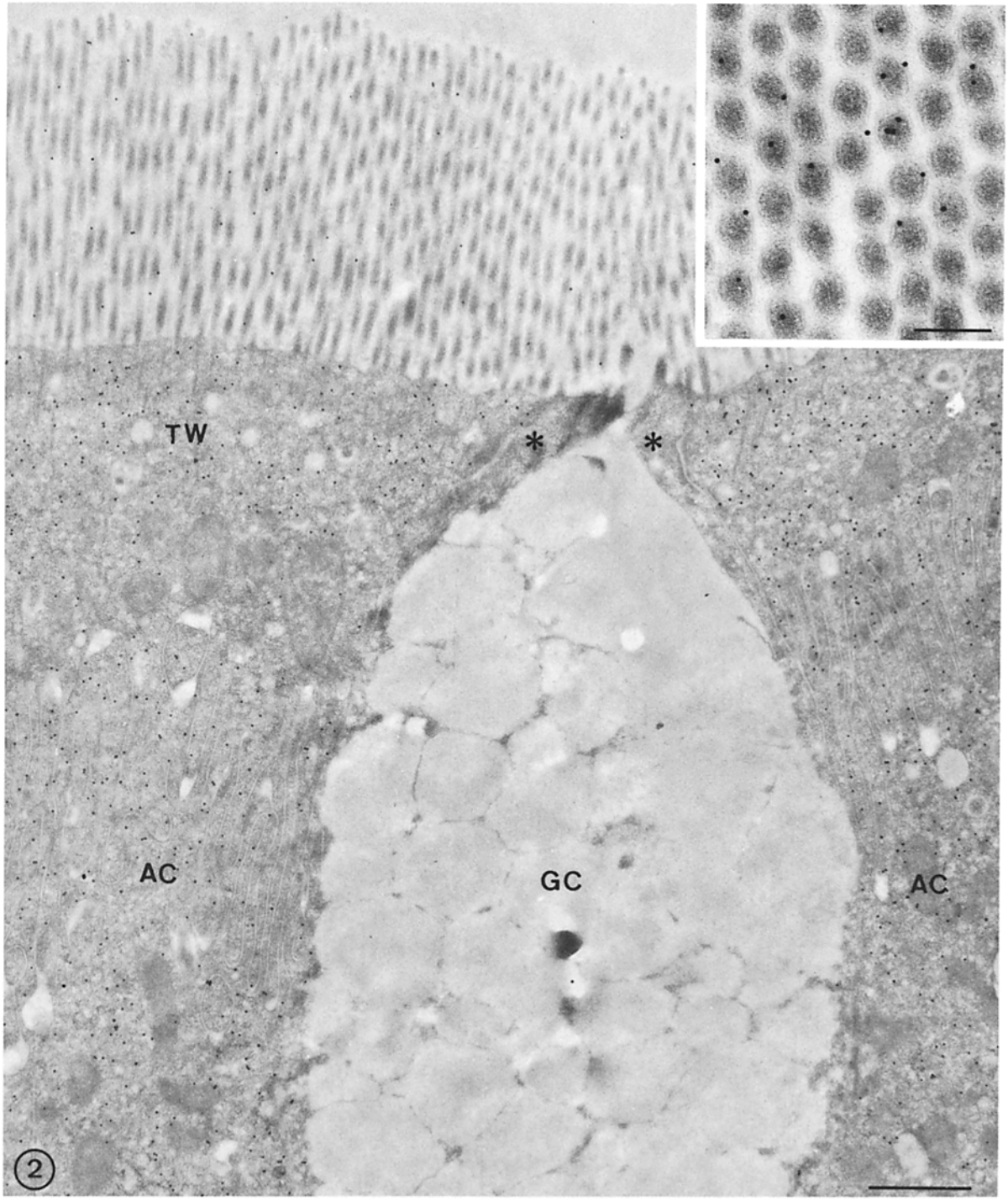


FIGURE 2 Figs. 2–7 are electron micrographs of chick duodenum fixed in glutaraldehyde and embedded at low temperature in Lowicryl K4M. Thin sections were stained for CaBP with the pAg technique. Fig. 2 shows apical region of two absorptive cells (*AC*) and of a goblet cell (*GC*). Gold particles revealing CaBP immunoreactive sites are present only over the absorptive cells, whereas the mucous droplets and a small peripheral rim of cytoplasm (asterisks) of the goblet cell are free of immunolabel. The terminal web region (*TW*) of the absorptive cells is densely labeled, whereas small, clear cytoplasmic vesicles, mitochondria, and lysosomes are negative. The brush border is sparsely labeled and the distribution of gold particles over cross-sectioned microvilli is shown at high magnification in the inset. Bar, 1 μm. × 16,500. *Inset*: Bar, 0.2 μm. × 62,000.

nmol of cholecalciferol orally, to convert them to a normal vitamin D-replete state, for 3 days before killing. After killing, the intestines were slit longitudinally, rinsed in PBS, and the duodenal mucosa was scraped with microscopic slides from the underlying serosa. A homogenate (10% wt/vol) was prepared in 0.25 M sucrose-TKM buffer (0.25 M sucrose-50 mM Tris-25 mM KCl-5 mM $MgCl_2$, pH 7.5) by 8–10 passes with a glass-teflon motor-driven homogenizer. A crude nuclear pellet was obtained by centrifugation at 750 *g* for 10 min, a crude mitochondrial pellet by centrifugation at 8,500 *g* for 20 min, and a microsomal pellet by centrifugation of the resulting supernatant at 105,000 *g* for 90 min. The final highspeed supernatant was designated cytosol. These centrifugation procedures are described in more detail by Wecksler et al. (46). A chromatin fraction was prepared from the crude nucleus pellet by the procedure of Haussler et al. (19).

Purified chick intestinal brush borders and brush border membrane fractions were prepared exactly as described by Putkey et al. (30).

All subcellular fractions were resuspended in 0.01 M sodium phosphate - 0.15 M NaCl, vigorously and repeatedly spun, and serial dilutions were evaluated for CaBP content by RIA.

RESULTS

Light Microscopy

As shown in Fig. 1 *a*, the specific fluorescence for CaBP was localized in the absorptive columnar epithelial cells of the villi; the goblet cells were negative. The intensity of fluorescence varied from one cell to another but the brush border region appeared always unstained. No CaBP fluorescence could be detected in the cells of the crypts (cf. Fig. 1 *a*). The specific fluorescence was completely abolished in sections incubated in antigen-absorbed antiserum.

Electron Microscopy

Unlike several other antigens demonstrated previously with the protein A-gold (pAg) technique on thin sections of glutaraldehyde-fixed and Epon-embedded tissues (3, 31–35) including CaBP in chick kidney (35), intestinal CaBP was undetectable on Epon sections of the duodenum. A specific labeling for CaBP was, however, observed in thin sections of glutaraldehyde-fixed, low temperature Lowicryl K4M-embedded duodenum. As in immunofluorescence-stained paraffin sections, CaBP was found only in the absorptive cells of the villi by this technique (Fig. 2). The other cell types of the mucosa, including

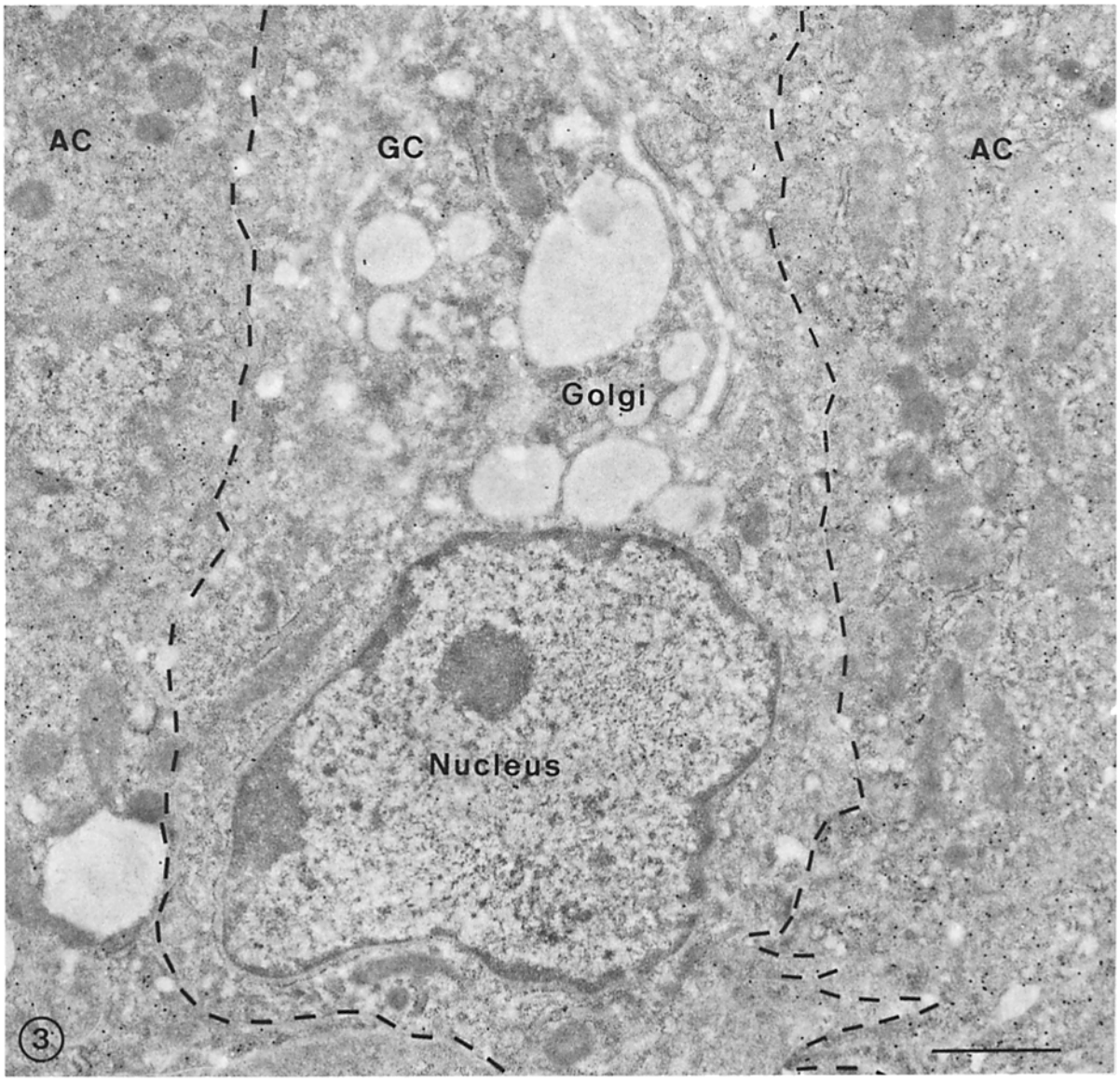


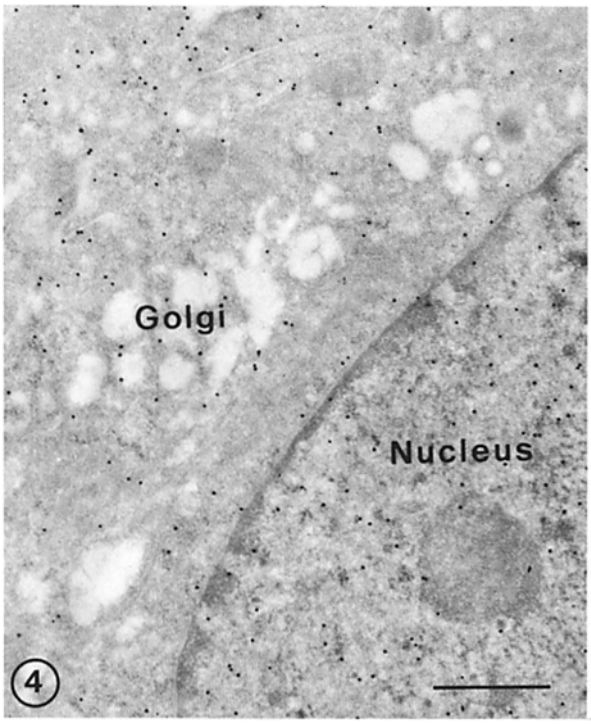
FIGURE 3 Basal region of two positive absorptive cells (*AC*) and of a negative goblet cell (*GC*, outlined with a dashed line). The gold particle label for CaBP is present only over the cytoplasmic matrix (cytosol) of the absorptive cells. Very low background label is seen over the goblet cell. Bar, 1 μm. × 16,500.

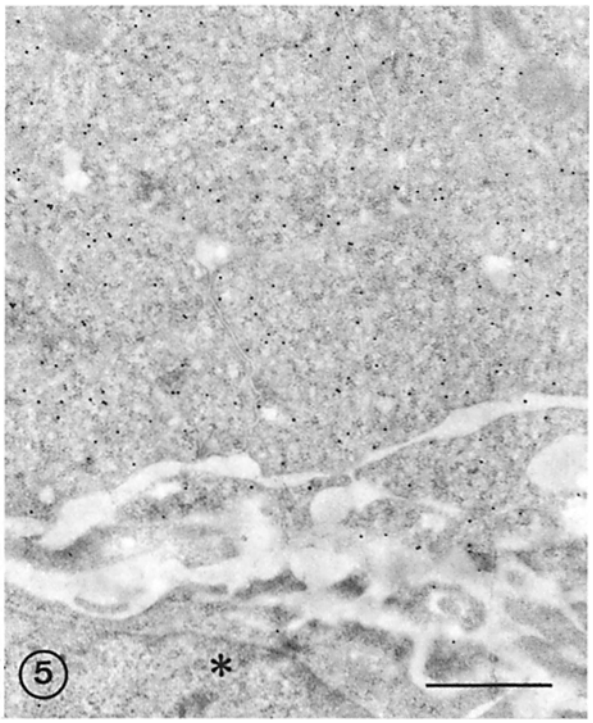
goblet cells (Figs. 2 and 3), endocrine cells, and cells migrating through the epithelium such as lymphocytes did not show a degree of gold labeling above background. In the absorptive cells, gold particles were scattered over the cytoplasmic matrix (cytosol), while none of the membrane-bounded intracellular compartments (except the nucleus, see below) was labeled (Figs. 2–6). The microvilli of the brush border showed a weak labeling (Fig. 2), the highest degree of labeling being present over the terminal web (Fig. 2). The nucleus was also marked by gold particles which were preferentially concentrated over the euchromatic zones (Figs. 4 and 7).

Table I shows the quantitative evaluation of the gold particle labeling for CaBP over the various cell regions described above. The values obtained confirm that the labeling over the absorptive cell is heterogeneous, the most highly labeled area of the cell being the terminal web, followed by the basal cytosol. The brush border had a lower number of gold particles, yet significantly above background, as had the euchromatin of the nucleus. Control Lowicryl K4M sections exposed to antigen-absorbed anti-CaBP or treated with nonimmune rabbit serum before incubation with the pAg solution showed a low degree of nonspecific background staining.

Radioimmunoassay

Table II presents the data concerning the subcellular distribution of the vitamin D-dependent CaBP in the chick duodenum as evaluated by radioimmunoassay. By far the highest concentration of CaBP is in the soluble or cytosol fraction of the cell. The cytosol CaBP in nanograms per milligram of protein was 82 times higher than in the crude nuclear and mitochondrial pellets which had the second highest CaBP concentration. In addition, 96% of the total CaBP of the whole homogenate was recovered in the cytosol. Only very low levels





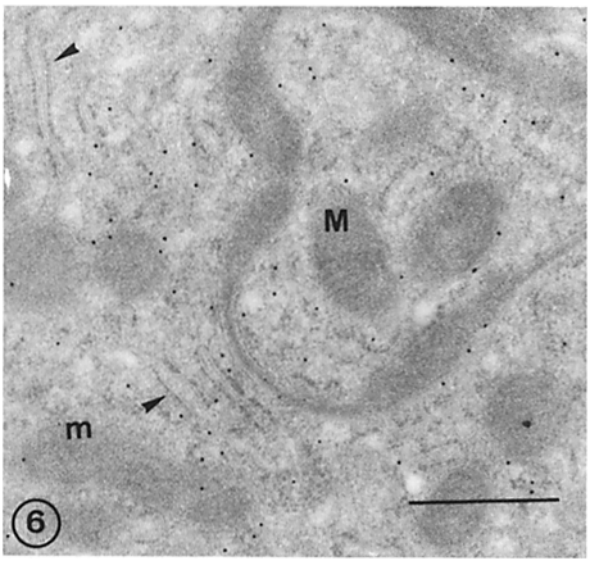


FIGURE 4. Perinuclear region of an absorptive cell with a Golgi apparatus and part of the nucleus. Label for CaBP is present over the cytosol and the euchromatin region of the nucleus. Cisternal spaces of the Golgi apparatus and clear cytoplasmic vesicles, nucleolus, and heterochromatin show low degree of background. Bar, 0.5 μm. × 31,000.

FIGURE 5. Basal part of absorptive cells with labeled cytosol. There is no preferential association of gold particles with the basolateral plasma membrane. No specific labeling is present over a fibroblast (asterisks) in the villus stroma or over the extracellular space. The few gold particles represent low background staining. Bar, 1 μm. × 17,000.

FIGURE 6. Detail of the cytoplasm from an absorptive cell showing gold particles over the cytosol but not over mitochondria (M) or the cisternal space of the rough endoplasmic reticulum (arrowheads). Bar, 1 μm. × 20,500.

of CaBP were detected in other subcellular membrane fractions, e.g. mitochondria, brush borders or microsomes. While the apparent concentration of CaBP in the nuclear fraction is low (16 ng/mg protein), due to the high total protein concentration of this subcellular fractions, the nuclear pellet has the second highest proportion of the total CaBP.

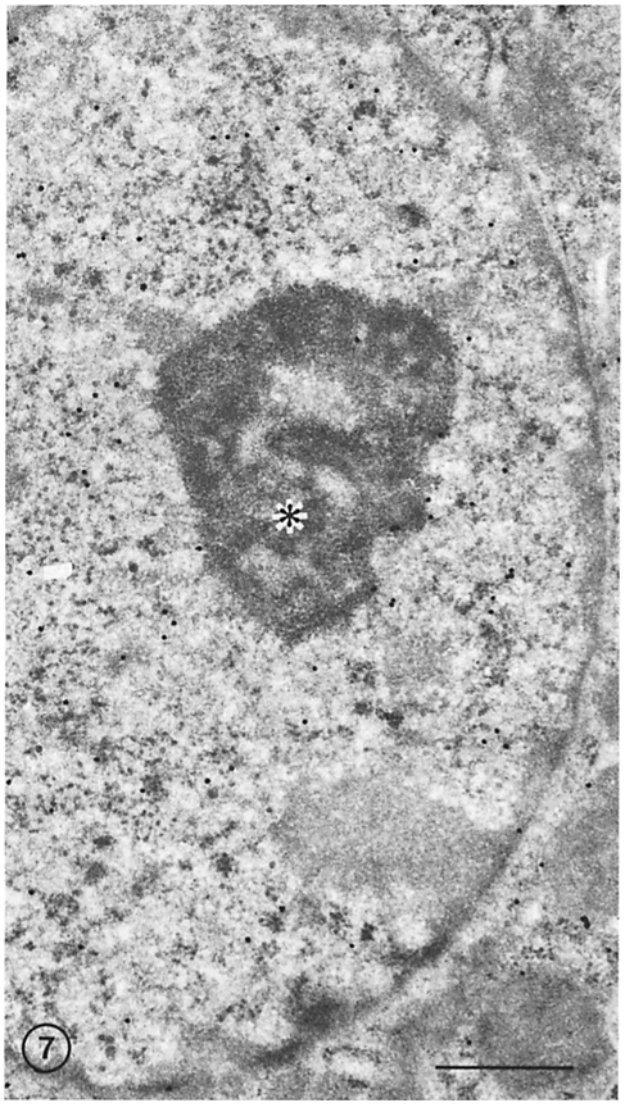


FIGURE 7 Detail of the nucleus from an absorptive cell. Specific label is over the euchromatin whereas the single gold particles over the nucleolus (asterisks) and heterochromatin corresponds to a low degree of background staining. Bar, 0.5 μm. × 34,000.

DISCUSSION

The vitamin D-dependent CaBP in chick duodenum was localized immunocytochemically and specific staining was found only in the differentiated absorptive columnar epithelial cells of the villi. This localization corresponds to that previously reported by others (1, 21, 25, 26, 39), and a recent reinvestigation by Taylor (39) has shown that his first reported localization of the CaBP to goblet cells is a relocation artifact which occurred during tissue section processing. Our results show in addition that differentiated absorptive cells (on the villi) differ from nondifferentiated cells (in the crypts) by their content of CaBP, although both possess receptors for $1,25(OH)_2D_3$ (38). This suggests that the presence of $1,25(OH)_2D_3$ receptors alone in intestinal columnar cells is not sufficient for the expression of $1,25(OH)_2D_3$ receptor-mediated transcriptional processes for CaBP.

Since CaBP is a water-soluble antigen (7) our finding of a cytosolic and nuclear (euchromatic) localization of CaBP in the columnar absorptive cell also deserves comment. We believe that this localization probably reflects the true distribution of CaBP rather than an artifactual redistribution, for the following reasons: (*a*) glutaraldehyde rapidly penetrates epithelia and cross-links cellular proteins, and fixation with higher concentrations of glutaraldehyde (2%) or short-time (30 min) mild fixation with 2% formaldehyde gives the same results; (*b*) low temperature embedding appears to prevent disruption of

TABLE II

Subcellular Distribution of Vitamin D-dependent CaBP in Chick Duodenum as Determined by Radioimmunoassay

Cell fraction	Total protein	CaBP	CaBP	CaBP*
	mg	*ng per fraction*	*ng/mg protein*	*%*
Homogenate	607	294,395	485 ± 30	
Nuclear (crude)	289	4624	16 ± 2	0.2
Chromatin	62	186	3 ± 1	–
Mitochondria (crude)	27	513	19 ± 2	–
Microsomes	32	128	4 ± 1	–
Cytosol	181	284,170	1,570 ± 210	96.5
	591 total recovered			
Brush border			210 ± 25	
Brush border membrane			14 ± 4	

* Calculated as CaBP (ng/mg protein) multiplied by total protein in that subcellular fraction divided by the total amount of CaBP measured in the homogenate.

TABLE I

Intensity of Immunolabeling for Vitamin D-dependent CaBP in Duodenal Columnar Epithelial Cells and Goblet Cells *

	Columnar cells					Goblet cells
	Brush border	Terminal web	Basal cytosol	Supranuclear cytosol	Euchromatin	Apical cytoplasm
			$P < 0.005$			
Anti-CaBP	7.23 ± 3.40	56.15 ± 2.02	50.56 ± 2.35	40.76 ± 2.23	43.37 ± 1.86	2.85 ± 0.54
	$P < 0.0001$					
Antigen-absorbed anti-CaBP	3.82 ± 0.90	6.60 ± 0.72	5.86 ± 0.56	7.86 ± 0.61	6.51 ± 0.44	2.74 ± 0.37
Normal serum	3.01 ± 0.28	4.35 ± 0.28	4.93 ± 0.28	4.74 ± 0.56	4.26 ± 0.49	2.30 ± 0.30

* Gold particles per square micrometer (±SEM).

supramolecular structures, hinders dislocation of proteinous cellular constituents, and results in excellent preservation of cellular fine structural details (8); (*c*) the cytosolic localization of CaBP correlates well with biochemical data showing CaBP synthesis on free ribosomes (10, 13, 36, 37) and its easy release upon tissue homogenization (41, 45); (*d*) RIA evaluation of the CaBP of isolated subcellular organelles from the chick duodenum (see Table II) suggests that the nuclei, but not chromatin, contain significant levels of CaBP; (*e*) $1,25(OH)_2D_3$ receptors are absent from goblet cells (38), and we did not detect CaBP in this cell type.

The gradient of cytosolic labeling which was maximal at the level of the terminal web and basal cytoplasm is also considered as an intrinsic pattern of CaBP distribution since this heterogeneous labeling was consistently observed in all intestinal samples examined. A further argument is that, with an entirely different fixation procedure, a preferential fluorescence was present in the region of terminal web (23). There is an apparent discrepancy between the quantitative cytochemical data and the RIA data. This is explained by methodological differences. Purified chromatin is composed mainly of DNA and has lost considerable amounts of its protein, phospholipid, and RNA during the preparative steps (19). Cytochemically, nuclear CaBP was found only in the euchromatin and, therefore, was quantitatively estimated only in relation to euchromatin. On the contrary, measurement of CaBP by RIA is proportional to the total protein present in the crude nuclear fraction.

Thus, the results of the present immunocytochemical study on subcellular localization of intestinal CaBP do not support a role of this vitamin D-dependent protein in the initial uptake and movement of Ca^{2+} across the brush border membrane of the epithelial cell as was suggested in earlier microscopical (42) and biochemical studies (11, 12, 14). Biochemical data which contradict an involvement of CaBP in Ca^{2+} transport across membranes include: (*a*) in rachitic chicks given $1,25(OH)_2D_3$, stimulation of in vitro Ca^{2+} transport occurs before CaBP specific messenger RNA and CaBP synthesis is initiated (5, 36, 37); (*b*) the lack of effect of inhibitors of protein and mRNA synthesis on Ca^{2+} transport across cellular membranes (15, 47) or on Ca^{2+} accumulation by mitochondria (28). Taken together, these biochemical and the present morphological data are compatible with the hypothesis that the intestinal vitamin D-dependent CaBP functions as a regulator of intracellular Ca^{2+} concentration. Such a function could involve the prevention of cytosolic and nuclear accumulation of free Ca^{2+} and/or the regulation of exchange of Ca^{2+} between the cytosolic and mitochondrial pool (6, 18). Alternatively, the CaBP may be involved in some aspects of the movement of Ca^{2+} across and its exit at the baso-lateral plasma membrane of the epithelial cells.

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REFERENCES

1. Arnold, B. M., K. Kovacs, and T. M. Murray, 1976. Cellular localization of intestinal calcium binding protein in pig duodenum. *Digestion* 14:77–84.
2. Bar, A., W. Hunziker, M. R. Walters, and A. W. Norman. 1981. Relationship between plasma $1,25(OH)_2D_3$ and its intestinal receptors and calcium binding protein levels as influenced by dietary calcium or phosphorus restriction. *Biochim. Biophys. Acta.* In press.
3. Bendayan, M., J. Roth, A. Perrelet, and L. Orci. 1980. Quantitative immunocytochemical localization of pancreatic secretory proteins in subcellular compartments of the rat acinar cells. *J. Histochem. Cytochem.* 28:149–160.
4. Biberfeld, P., V. Ghetie, and J. Sjöquist. 1975. Detection and assaying of IgG antibodies in tissues and on cells by labeled staphylococcal protein A. *J. Immunol. Methods* 6:249–259.
5. Bikle, D. D., D. T. Zolok, R. L. Morrissey, and R. H. Herman. 1978. Independence of 1,25-dihydroxyvitamin D_3-mediated calcium transport from de novo RNA and protein synthesis. *J. Biol. Chem.* 253:484–488.
6. Bikle, D. D., E. W. Askew, D. T. Zolok, R. L. Morrissey, and R. H. Herman. 1980. Calcium accumulation by chick intestinal mitochondria. Regulation by vitamin D_3 and 1,25-dihydroxyvitamin D_3. *Biochim. Biophys. Acta.* 598:561–574.
7. Bredderman, P. J., and R. H. Wasserman. 1974. Chemical composition, affinity for calcium, and some related properties of the vitamin D-dependent calcium binding protein. *Biochemistry.* 13:1687–1694.
8. Carlemalm, E., M. Garavito, and W. Villiger. 1982. Resin development for electron microscopy and analysis of embedding at low temperature. *J. Microsc. (Oxf.).* In press.
9. Christakos, S., E. J. Friedlander, B. R. Frandsen, and A. W. Norman. 1979. Studies on the mode of action of calciferol. XIII. Development of a radioimmunoassay for vitamin D-dependent chick intestinal calcium-binding protein and tissue distribution. *Endocrinology.* 104:1495–1503.
10. Christakos, S., and A. W. Norman. 1980. Apparent messenger RNA activity for vitamin D-dependent calcium binding protein in chick kidney and duodenum. *Arch. Biochem. Biophys.* 203:809–815.
11. Corradino, R. A., C. S. Fullmer, and R. H. Wasserman. 1976. Embryonic chick intestine in organ culture: stimulation of calcium transport by exogenous vitamin D-induced calcium binding protein. *Arch. Biochem. Biophys.* 174:738–743.
12. Ebel, J. G., A. N. Taylor, and R. H. Wasserman. 1969. Vitamin D-induced calcium binding protein of intestinal mucosa. Relation to vitamin D dose level and lag period. *Am. J. Clin. Nutr.* 22:431–436.
13. Emtage, J. S., D. E. M. Lawson, and E. Kodicek. 1973. Vitamin D-induced synthesis of RNA for calcium binding protein. *Nature (Lond.).* 246:100–101.
14. Emtage, J. S., D. E. M. Lawson, and E. Kodicek. 1974. The response of the small intestine to vitamin D. Correlation between calcium-binding protein production and increased calcium absorption. *Biochem. J.* 144:339–346.
15. Franceschi, R. T., and H. F. De Luca. 1981. The effect of inhibitors of protein and RNA synthesis on $1\alpha,25$-dihydroxyvitamin D_3-dependent calcium uptake in cultured embryonic chick duodenum. *J. Biol. Chem.* 256:3848–3852.
16. Frens, G. 1973. Controlled nucleation for the regulation of particle size in monodisperse gold solutions. *Nature Phys. Sci.* 241:20–22.
17. Friedlander, E. G., H. L. Henry, and A. W. Norman. 1977. Effects of dietary calcium and phosphorus on the relationship between the 25-hydroxyvitamin D_3-1α-hydroxylase and production of chick intestinal calcium binding protein. *J. Biol. Chem.* 252:8677–8683.
18. Hamilton, J. W., and E. S. Holdsworth. 1970. The release of ^{45}Ca from mitochondria of chicken intestinal mucosa by calcium binding protein. *Biochem. Biophys. Res. Commun.* 40:1325–1330.
19. Haussler, M. R., W. W. Thomson, and A. W. Norman. 1969. The isolation of purified nuclear and chromatin fractions from chick intestinal mucosa. *Exp. Cell Res.* 58:234–242.
20. Hunziker, W., M. E. Waltzer, J. E. Bishop, and A. W. Norman. 1982. Effects of vitamin D status on the equilibrium between occupied and unoccupied receptors. *J. Clin. Invest.* 69:826–834.
21. Jande, S. S., S. Tolnai, and D. E. M. Lawson. 1981. Immunohistochemical localization of vitamin D-dependent calcium-binding protein in duodenum, kidney, uterus, and cerebellum of chickens. *Histochemistry.* 71:99–116.
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
23. Marche, P., P. Cassier, and H. Mathieu. 1980. Intestinal calcium-binding protein. A protein indicator of enterocyte maturation associated with the terminal web. *Cell Tissue Res.* 212:63–72.
24. Morrissey, R. L., and R. H. Wasserman. 1971. Calcium absorption and calcium-binding protein in chicks on differing calcium and phosphorus intakes. *Amer. J. Physiol.* 220:1509–1515.
25. Morrissey, R. L., D. T. Zolok, T. J. Bucci, and D. D. Bickle. 1978. Immunoperoxidase localization of vitamin D-dependent calcium binding protein. *J. Histochem. Cytochem.* 26:628–634.
26. Morrissey, R. L., R. N. Empson, Jr., D. T. Zolok, D. D. Bickle, and T. J. Bucci. 1978. Intestinal response to $1\alpha,25$-dihydroxycholecalciferol. II. A timed study of the intracellular localization of calcium binding protein. *Biochim. Biophys. Acta.* 538:34–41.
27. Morrissey, R. L., D. T. Zolok, P. W. Mellick, and D. D. Bickle. 1980. Influence of cycloheximide and 1,25-dihydroxyvitamin D_3 on mitochondrial and vesicle mineralization in the intestine. Cell Calcium 1:69–79.
28. Norman, A. W. 1979. Vitamin D: Calcium Homeostatic Steroid Hormone. W. F. Darby, editor. Academic Press, Inc., New York 1–490.
29. Norman, A. W., and R. G. Wong. 1972. The biological activity of the vitamin D metabolite, 1,25-dihydroxycholecalciferol in chickens and rats. *J. Nutrition.* 102:1709–1718.
30. Putkey, J. A., A. M. Spielvogel, R. S. Sauerheber, C. S. Dunlap, and A. W. Norman. 1982. Vitamin D-mediated intestinal calcium transport: effects of essential fatty acid deficiency and spin label studies of enterocyte membrane lipid fluidity. *Biochim. Biophys. Acta.* In press.
31. Roth, J. 1982. The protein A-gold (pAg) technique. Qualitative and quantitative approach for antigen localization on thin sections. *In*: Immunocytochemical Techniques and Applications. G. R. Bullock and P. Petrusz, editors. Academic Press, Inc., London. 107–135.
32. Roth, J., M. Bendayan, and L. Orci. 1978. Ultrastructural localization of intracellular antigens by the use of protein A-gold complex. *J. Histochem. Cytochem.* 26:1074–1081.
33. Roth, J., M. Bendayan, and L. Orci. 1980. FITC-protein A-gold complex for light and electron microscopic immunocytochemistry. *J. Histochem. Cytochem.* 28:55–57.
34. Roth, J., M. Ravazzola, M. Bendayan, and L. Orci. 1981. Application of the protein A-gold technique for electron microscopic demonstration of polypeptide hormones. *Endocrinology.* 108:247–253.
35. Roth, J., B. Thorens, W. Hunziker, A. W. Norman, and L. Orci. 1981. Vitamin D-dependent calcium binding protein: immunocytochemical localization in chick kidney. *Science (Wash. D. C.).* 214:197–200.
36. Spencer, R., M. Charman, P. W. Wilson, and D. E. M. Lawson. 1976. Vitamin D-stimulated intestinal calcium absorption may not involve calcium-binding protein directly. *Nature (Lond.).* 263:161–163.

37. Spencer, R., M. Charman, P. W. Wilson, and D. E. M. Lawson. 1978. The relationship between vitamin D-stimulated calcium transport and intestinal calcium-binding protein in chicken. *Biochem. J.* 170:93–101.
38. Stumpf, W. E., M. Sar, F. A. Reid, Y. Tanaka, and H. F. De Luca. 1979. Target cells for 1,25-dihydroxyvitamin D_3 in intestinal tract, stomach, kidney, skin, pituitary, and parathyroid. *Science (Wash. D. C.).* 206:1188–1190.
39. Taylor, A. N. 1981. Immunocytochemical localization of the vitamin D-induced calcium-binding protein: relocation of antigen during frozen section processing. *J. Histochem. Cytochem.* 29:65–73.
40. Taylor, A. N., and R. H. Wasserman. 1965. A vitamin D_3-dependent factor influencing calcium binding by homogenates of chick intestinal mucosa. *Nature (Lond.).* 205:248–250.
41. Taylor, A. N., and R. H. Wasserman. 1967. Vitamin D_3-induced calcium-binding protein: partial purification, electrophoretic visualization, and tissue distribution. *Arch. Biochem. Biophys.* 119:536–540.
42. Taylor, A. N., and R. H. Wasserman. 1970. Immunofluorescent localization of vitamin D-dependent calcium binding protein. *J. Histochem. Cytochem.* 18:107–115.
43. Wasserman, R. H., and A. N. Taylor. 1963. Vitamin D_3 inhibition of radiocalcium binding by chick intestinal homogenates. *Nature (Lond.).* 198:30–32.
44. Wasserman, R. H., and A. N. Taylor. 1966. Vitamin D_3-induced calcium-binding protein in chick intestinal mucosa. *Science (Wash. D. C.).* 152:791–793.
45. Wasserman, R. H., R. A. Corradino, and A. N. Taylor. 1968. Vitamin D-dependent calcium-binding protein: purification and some properties. *J. Biol. Chem.* 243:3978–3986.
46. Wecksler, W. R., H. L. Henry, and A. W. Norman. 1977. Subcellular localization of 1,25-dihydroxyvitamin D_3 in chicken parathyroid glands. *Arch. Biochem. Biophys.* 183:168–175.
47. Zolok, D. T., D. D. Morrissey, and D. D. Bickle. 1977. The effect of $1,25(OH)_2D_3$ on calcium accumulation, calcium transport, and calcium binding protein in the presence and absence of cycloheximide. *In:* Vitamin D: Biochemical, Chemical, and Clinical Aspects related to Calcium Metabolism. A. W. Norman, K. Schaefer, J. W. Coburn, H. F. De Luca, D. Fraser, H. G. Grigoleit, and D. v. Herrat, editors. Walter de Gruytr, Inc. New York, 345–347.